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Title: Non-heme Iron Oxygenase Catalysts

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Defense Priority Rating: N/A

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RESTRICTIONS

See Attached NIH Supplemental Information Sheet for Additional Requirements.

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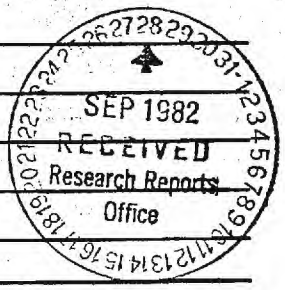
Equipment: Title vests with G.I.T. We are accountable for all equipment purchased.

COMMENTS: Follows on To G33-H05/May



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SPONSORED PROJECT TERMINATION/CLOSEOUT SHEET

Date April 25, 1985

Project No. G-33-H06 School/~~Lab~~ Chemistry

Includes Subproject No.(s) N/A

Project Director(s) S.W. May ~~EXXX~~/ GIT

Sponsor ~~DHHS~~/PHS/NIH-National Institute of General Medical Sciences

Title Non-Heme Iron Oxygenase Catalysts

Effective Completion Date: 3/31/84 (Performance) 6/30/84 (Reports)

Grant/Contract Closeout Actions Remaining:

- [ ] None
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APPENDIX 3

PROTOCATECHUATE 3,4-DIOXYGENASE: COMPARATIVE STUDY OF INHIBITION AND ACTIVE-SITE INTERACTIONS OF PYRIDINE N-OXIDES ‡

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Running Title: Protocatechuate dioxygenase inhibition

FOOTNOTES

[1]The abbreviations used are PCA, protocatechuic acid; PCD, protocatechuate 3,4-dioxygenase (protocatechuate: oxygen 3,4-oxidoreductase, EC 1.13.11.3); HINANO, 2-hydroxyisonicotinic acid N-oxide; 3-FHB, 3-fluoro-4-hydroxybenzoic acid; 3-ClHB, 3-chloro-4-hydroxybenzoic acid; ClNANO, 2-chloroisonicotinic acid N-oxide; NANO, nicotinic acid N-oxide; INANO, isonicotinic acid N-oxide; MINANO, 2-mercaptoisonicotinic acid N-oxide; 3-XHB, 3-halo-4-hydroxybenzoic acid.

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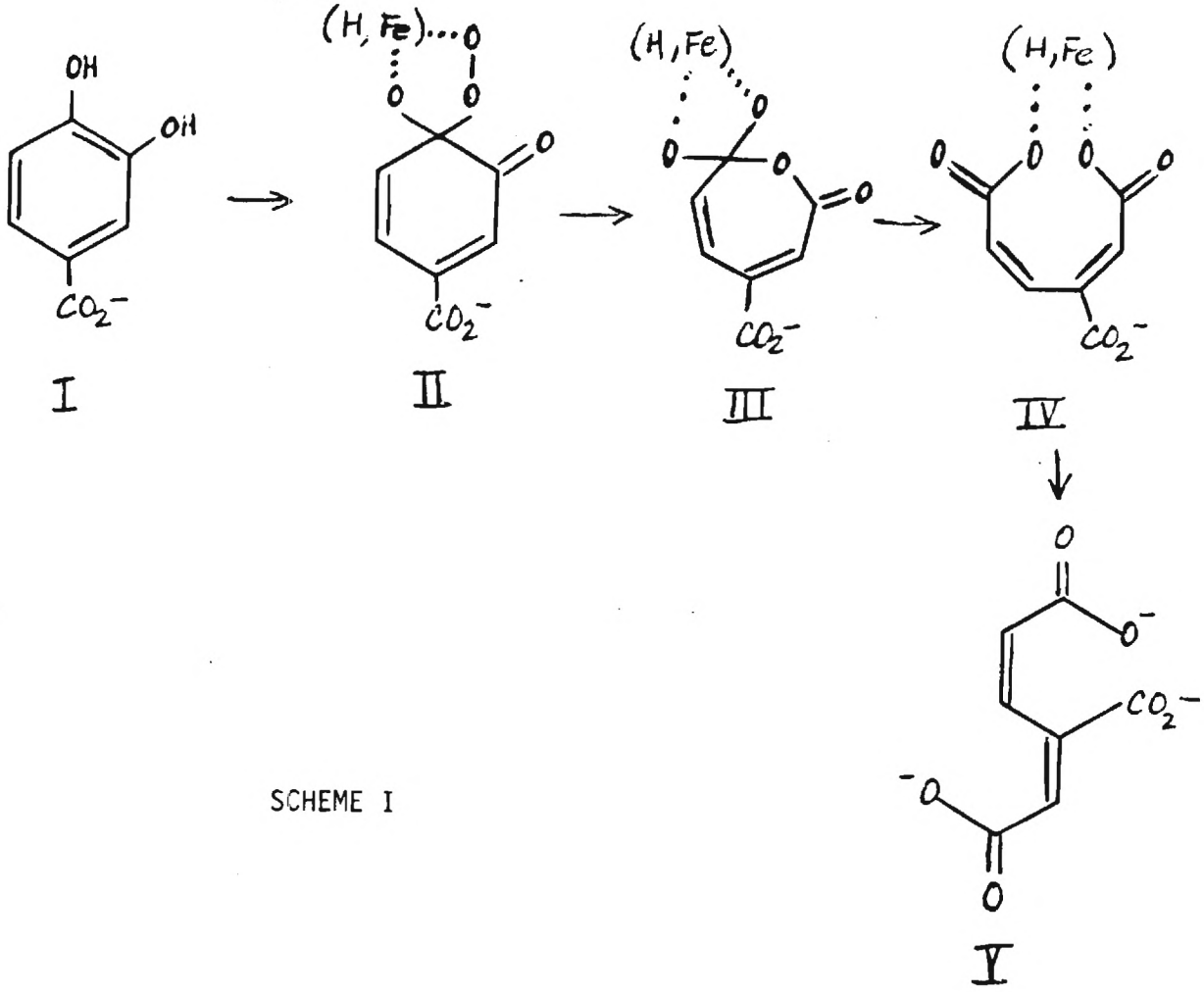
ABSTRACT

The binding of 2-hydroxypyridine N-oxide (HYPNO) and a series of structural analogs to protocatechuate 3,4-dioxygenase (PCD) has been investigated using kinetic and spectral techniques. HYPNO binds less tightly to PCD than 2-hydroxyisonicotinic acid N-oxide (HINANO), a compound designed to mimic structural features of species along the PCD reaction pathway, which we have previously shown to bind to the enzyme in a kinetically irreversible manner [J. Biol. Chem. 257, 12746-12751 (1982)]. HYPNO was found to be a time-dependent inhibitor of PCD. The rate constants for both binding (100 M^{-1} sec^{-1}) and dissociation (3×10^{-4} sec^{-1}) of HYPNO were found to be four orders of magnitude less than those for 3-fluoro-4-hydroxybenzoic acid (3-FHB) the best freely reversible competitive inhibitor of PCD. Although inhibition by HYPNO is not freely reversible, it can be slowly reversed by a simple displacement with 3-FHB. This is in contrast to the inhibition by HINANO whose binding to PCD is reversed only by denaturing the enzyme. The displacement of HYPNO by 3-FHB was examined spectrophotometrically and found to consist of rapid formation of a species which is spectrally distinct from the complex of PCD with either inhibitor alone, followed by a slow change to give the spectrum of the PCD·3-FHB complex. Analysis of the inactivation and spectral data, along with determination of the concentrations of both inhibitors immediately after the initial rapid change, is consistent with formation of a ternary PCD·HYPNO·3-FHB complex. The initial binding and time-dependence of inhibition of a series of related ring substituted pyridine N-oxides were also studied. The presence of a ketonizable group adjacent to the N-oxide causes slow, tight binding of the type seen with HYPNO and HINANO. The spectral characteristics of the complexes of HYPNO and HINANO with PCD differ markedly from those of the halohydroxybenzoate·PCD complexes and this may reflect differences in the ligation environment of the active site iron in these species.

INTRODUCTION

Among the reactions catalyzed by dioxygenases is the oxygenolytic cleavage of aromatic rings, and the mechanism of this process is a subject of much current interest. One of these enzymes, protocatechuate 3,4-dioxygenase (PCD)[1], which catalyzes the intradiol cleavage of protocatechuic acid, has been isolated from a number of microbial genera (Stanier & Ingraham, 1954; Wells, 1972; Hou et al., 1976; Durham et al., 1980; Bull and Ballou, 1981), with the crystalline enzyme from Pseudomonas aeruginosa being the most extensively studied (Fujisawa & Hayaishi, 1968). One method of investigating the enzymatic mechanism is by studying the interaction of various types of inhibitors with the enzyme active site. We, and others, have studied the 3-halo-4-hydroxybenzoates and have found them to be potent rapidly reversible PCD inhibitors (May et al., 1978; May & Phillips, 1979; Felton et al., 1978; Fujisawa et al., 1971; Fujisawa et al., 1972a; Fujisawa et al., 1972b; Que et al., 1977; Keyes et al., 1978; Tatsuno et al., 1978; Nakata et al., 1978). When complexed with these inhibitors, the enzyme gives a characteristic spectrum with a visible maximum at 420 nm. Kinetic and resonance Raman investigations carried out in this and other laboratories, have provided evidence that in the initial binding step, the p-OH of substrates or inhibitors interacts directly with the essential iron atom of the enzyme via Fe-O ligation. In the case of substrates, subsequent ketonization of the m-OH allows oxygen attack at the adjacent carbon to give an α-ketohydroperoxide, followed by collapse to product (Scheme I).

In order to obtain further support for this mechanism we have recently studied a compound designed to mimic the ketonized transient species, (VI) along the reaction pathway (May et al., 1982). This compound, 2-hydroxyisonicotinic acid N-oxide (HINANO, VIIa) was found to be an extremely potent, kinetically irreversible inhibitor and an active site titrant, although it is not a substrate for PCD. Furthermore, denaturation of the enzyme·HINANO complex released virtually all of the bound inhibitor in an active form, indicating that covalent binding of HINANO

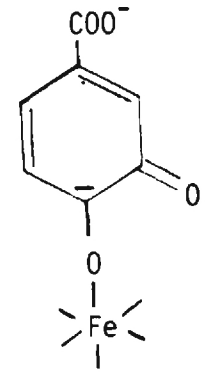


SCHEME I

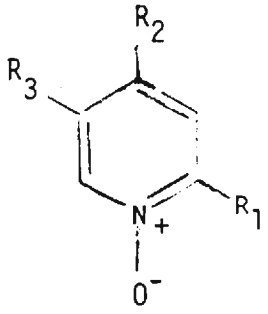
to the enzyme is unlikely. Inhibition with similar characteristics has been observed in studies with "transition state analogs" of various enzymes (Frieden et al., 1980; Wolfenden, 1976). The spectrum of the enzyme•HINANO complex was found to resemble that reported by Bull et al., (1981) for an "ESO_2" transient oxygenated intermediate detected in stopped-flow studies under hyperbaric oxygen conditions.

A detailed kinetic description of the interaction of hydroxypyridine N-oxides with PCD would provide a basis for contrasting the binding characteristics of these compounds to those of ground state inhibitors and substrate analogs. However, kinetic analysis of the interaction of PCD with HINANO using standard techniques is not feasible due to the kinetic irreversibility of inhibition by

this compound. We have, therefore, undertaken a detailed study of the interaction of PCD with 2-hydroxypyridine N-oxide (HYPNO,VIId). HYPNO retains the structural resemblance to the transient species VI but lacks a para carboxylate. By analogy to substrate analogs and ground state inhibitors (Que et al., 1977) we therefore expected that HYPNO binding to PCD would exhibit a greater degree of reversibility, thus allowing a detailed kinetic analysis. Furthermore, we wished to examine various related pyridine N-oxides (VII b,c,e), in order to assess the effects of ring substituents on binding to the PCD active site. Our results provide insight into the details of the interaction of these compounds, and of halohydroxybenzoate inhibitors, with the active site PCD.



VI



VII

		R_1	R_2	R_3
a	HINANO	OH	COOH	H
b	INANO	H	COOH	H
c	CINANO	Cl	COOH	H
d	HYPNO	OH	H	H
e	NANO	H	H	COOH
f	MINANO	SH	COOH	H

MATERIALS AND METHODS

All commercial materials were the highest grade obtainable. Protocatechuic acid, HYPNO, nicotinic acide N-oxide (NANO), and 3-chloro-4-hydroxybenzoic acid (3-ClHB) were recrystallized from water. Isonicotinic acid N-oxide (INANO) was recrystallized from methanol. HINANO and 2-chloroisonicotinic acid N-oxide (CINANO) were synthesized and purified as described previously (May et al., 1982). 3-Fluoro-4-hydroxybenzoic acid (3-FHB) was synthesized as previously described (May et al.,1978). Protocatechuate 3,4-dioxygenase was isolated from 4-hydroxybenzoate-induced cells of Pseudomonas species ATCC 23975 as described previously (May et al., 1978).

Synthesis of 2-Mercaptoisonicotinic Acid N-oxide (MINANO)

MINANO was synthesized by the following procedure, based on the general method of Taylor and Driscoll(1960). Into a flask, previously evacuated and filled with argon, 8.85g (0.123 mol) of KHS (Alfa Chemical Co.) was weighed. The contents of the flask were kept under an atmosphere of argon throughout the experiment. The flask was fitted with a reflux condenser and 84 ml of methanol (absolute) was added. This addition resulted in a clear olive-green solution. At reflux,4.12g (.0238 mol) of purified 2-chloroisonicotinic acid N-oxide was added and a milky-lime colored mixture resulted. The reaction mixture was refluxed for 21 hours with continuous stirring. A mixture with a clear lime colored top layer, a middle layer of white solid, and a bottom layer of yellowish-green solid resulted. The remaining methanol was removed by vacuum distillation leaving a yellow and white solid. The solid was dissolved in 60 ml of distilled-deionized water. The resulting green solution was acidified with concentrated HCl and chilled at $4^{o}C$. The resulting yellow solid was then filtered with a buchner funnel. The crude solid was dissolved in a minimal amount of hot distilled deionized water and immediately filtered. The filtrate was stored overnight at $4^{o}C$. The yellow-gold crystals were collected on a

buchner funnel and vacuum dried. This recrystallization resulted in 1.64g of MINANO. The MINANO was shown to be pure by NMR (D_2O, 5% NaOD, 3-(trimethylsilyl)propionic acid, Na salt as internal standard: multiplet at 8.13δ, 2H; multiplet at 7.32δ, 1H); mass spectrum (m/e 171 molecular ion) and elemental analysis (calculated: C, 42.10%, H, 2.94%, N, 8.18%, S, 18.73%; found: C, 42.10%, H, 2.96%, N, 8.12%, S, 18.73%). The purified MINANO was stored under argon to prevent decomposition.

Dialysis of PCD·HYPNO Complex

A two ml sample of a solution containing PCD (320 nM) and HYPNO (91 μM) in 0.05 M Tris·HCl, pH 7.5, buffer was placed in an Amicon ultrafiltration unit (model 8MC) and dialyzed against 0.05 M Tris·HCl, pH 7.5, buffer until 20 ml of effluent were collected. The retentate was removed and the enzyme activity was assayed and compared with the activity of both the undialyzed PCD·HYPNO solution and the PCD solution before addition of HYPNO. The activity was measured spectrophotometrically by following the decrease in absorbance at 290 nm of a 0.4 mM protocatechuic acid solution in 0.05 M Tris·HCl buffer, pH 7.5 (Stanier & Ingraham, 1954).

Measurement of Apparent K_I's

The K_I's for HYPNO, NANO, INANO, and CINANO were determined by measuring PCD activity using a YSI Model 53 oxygen monitor equipped with a Clark polarographic electrode and thermostatted to 25°C. The decrease in dissolved oxygen with time was recorded and initial rates measured graphically. All inhibitors showed a slope effect on the double reciprocal plot and K_I values were estimated from the ratio of slopes of the inhibited and uninhibited data. The K_I for HINANO was measured spectrophotometrically using a stopped-flow apparatus to examine the initial rate of substrate oxidation at 290 nm as previously described (May et al., 1978; May & Phillips, 1979).

Calculation of First Order Rate Constants

A reiterative non-linear least squares program (Duggleby, 1981) was used to fit the data to the equation $\ln (A_\infty - A_t) = \ln (A_\infty - A_0) - kt$.

Spectral Studies of the Binding of HYPNO to PCD

The absorbance at 437 nm of an enzyme solution 11.76 μM in active sites in 0.05 M Tris·HCl, pH 7.5 at 25°C was followed upon the addition of HYPNO in ratios of 4:1, 10:1, and 20:1 HYPNO to active sites. Initial rates of change were calculated based on a $\Delta\varepsilon$ of 514.6 $M^{-1}cm^{-1}$ and second order rate constants were calculated from these initial rates.

Dialysis Binding Experiments

In these experiments the appropriate amount of enzyme was dialyzed against 0.05 M Tris·HCl, pH 7.5 at 25°C in an Amicon Ultrafiltration unit fitted with an XM100A membrane until the effluent contained no significant absorbance at the wavelengths of interest. The appropriate amount of HYPNO was then added to the enzyme solution and that solution immediately removed from the unit. The solution was diluted to volume with washes from the Amicon unit and incubated for 30 min at room temperature. The appropriate amount of 3-FHB or 3-ClHB was then added and the solution was immediately returned to the Amicon unit and filtered under pressure. The effluents were obtained 3 to 4 min after the addition of the halohydroxybenzoate inhibitor. The concentration of HYPNO and halohydroxybenzoate in the effluent were determined from absorbances at two different wavelengths by the solution of simultaneous equations. For HYPNO and 3-FHB, ε_{265}(HYPNO) = 388 $M^{-1}cm^{-1}$, ε_{265} (3-FHB) = 6210 $M^{-1}cm^{-1}$, ε_{315} (HYPNO) = 5121 $M^{-1}cm^{-1}$, and ε_{315} (3-FHB) = 94 $M^{-1}cm^{-1}$ were used. For HYPNO and 3-ClHB ε_{276} (HYPNO) = 677 $M^{-1}cm^{-1}$, ε_{276} (3-ClHB) = 7362 $M^{-1}cm^{-1}$, ε_{314} (HYPNO) = 5052 $M^{-1}cm^{-1}$, and ε_{314} (3-ClHB) = 625 $M^{-1}cm^{-1}$ were used. Since HYPNO binding has

reached equilibrium 30 min following the addition of HYPNO, it can be assumed that the solution species are E + HYPNO $\rightleftharpoons$ E·HYPNO. Upon addition of 3-FHB, equilibrium is rapidly established with the enzyme species . Since 3-FHB binds more rapidly to E than E·HYPNO (relative k_{on}'s), the enzyme species after addition of 3-FHB are E·3FHB, E·HYPNO.3FHB, and E·HYPNO when both inhibitors and enzyme active sites are present at equal concentrations. The 3-FHB concentration is measured directly, the [E·HYPNO] = [3-FHB], and [E·HYPNO·3FHB] = $[E]_{total}$ - [3FHB] - [HYPNO]. Therefore $K_D^{(2)}$'s can be calculated equal to $\frac{[3FHB][E\cdot HYPNO]}{[E\cdot HYPNO\cdot 3FHB]}$. The same method was used to calculate the K_D for the dissociation of 3-ClHB from the ternary complex.

$K_D^{(1)}$'s for the dissociation of HYPNO from E·HYPNO can also be calculated from the 1:1:1 dialysis data as the addition of halohydroxybenzoate does not affect the E, HYPNO equilibrium within the time of the experiment. The [HYPNO] can be measured directly and [E·HYPNO] assumed to be $[E]_{total}$ - [HYPNO]. Likewise, [E] is assumed to equal [HYPNO]. $K_D^{(1)}$ is therefore calculated according to the equation $K_D = \frac{[E][HYPNO]}{[E\cdot HYPNO]}$.

Henderson Plot

Initial rates were determined using stopped-flow where enzyme and inhibitors were preincubated 75 min before flowing against substrate. The stopped-flow accessory of a DW-2 spectrophotometer (Aminco) maintained at 25°C was used to mix 43 nM enzyme active sites inhibited by HYPNO after preincubating 75 min at ratios of 35:1 to 600:1 HYPNO:active sites with 60 μM PCA. Both solutions were in 0.05 M Tris·HCl, pH 7.5 at 25°C and final concentrations were one-half those stated. A_{290} was followed in the dual beam mode with the reference at 400 nm and initial rates of decrease were calculated using ε = 2280 $M^{-1}cm^{-1}$.

Spectrum of the Anaerobic PCD•HINANO Complex

A closed system was used consisting of a quartz cuvette containing 15 µl of PCD (149 µM) in 1 ml of 0.05M Tris•HCl pH 7.5 buffer, a sidearm containing 25 µl of 10 mM PCA and a buret containing HINANO (774 µM) in the same buffer. The system was made anaerobic by 6 cycles of evacuation and repressurizing with argon. After recording the spectrum of native enzyme, the substrate was tipped in and the anaerobic E•S complex was recorded. A total of 150 µl of anaerobic HINANO solution was added from the buret and the spectrum of the E•I complex was recorded after a 30 min wait. The system was opened, compressed air was bubbled through the E•I complex and the aerobic spectrum was also recorded.

RESULTS

Spectral Study of the Anaerobic PCD·HINANO Complex

We have previously reported that HINANO, which possesses all the essential elements of the postulated transient species (VI), is the most potent inhibitor known for PCD (May et al.,1982). Since the spectral change observed when HINANO binds to the enzyme is similar to an "ESO_2" intermediate reported by Bull et al. (1981) it seemed possible that it could reflect formation of a ternary EIO_2 complex. This possibility was tested by preparing the PCD·HINANO complex under both anaerobic and aerobic conditions as described in Materials and Methods.

In the anaerobic experiments, substrate was first added to the anaerobic PCD solution in order to confirm formation of the characteristic E·S spectrum which would not have been observed in the presence of oxygen (Fujisawa et al., 1971). HINANO was then added causing complete displacement of substrate and formation of the spectrum characteristic of the PCD·HINANO complex. It was evident that the spectrum of this complex is identical to that observed under aerobic conditions, thus establishing that the formation of a ternary PCD·HINANO·O_2 complex is not responsible for the characteristic spectral change.

Inhibition by Pyridine N-Oxides

Table I lists the apparent K_I's obtained for a series of pyridine N-oxides which differ in their pattern of ring substitution. The apparent K_I's listed were obtained under normal conditions for ground-state inhibitors, i.e., measurement of initial rates without preincubation of the enzyme and inhibitor. It is apparent from these values that lack of either the carboxylate or the ketonized m-hydroxyl causes a drastic decrease in binding affinity of more than 2 orders of magnitude. Furthermore, altering the para relationship of

the N-oxide and carboxylate functionalities also greatly reduces inhibitor potency. Unexpectedly, INANO is a better inhibitor than CINANO; whereas, the opposite is true for the halohydroxybenzoates (May & Phillips, 1979).

Taken together, the overall trends observed are in accord with what would be expected for compounds which mimic transient species along the reaction pathway, the essential features being carboxylate, p-oxygen available for iron ligation and ketonizable m-oxygen.

Time Dependence of Inactivation by Pyridine N-Oxides

During investigations of the time dependence of PCD inhibition by the pyridine N-oxides listed in Table I, it became immediately apparent that inhibition by HYPNO is not rapidly reversible. As shown in Figure 1A when PCD was preincubated with excess HYPNO and then diluted into an assay solution, a time dependent loss of PCD activity was observed. In these experiments the enzyme · HYPNO mixture was diluted 120 fold, and the assay mixture contained a 450 to 4500-fold excess of substrate, and thus rapidly reversible inhibition would not be seen. In contrast, when similar experiments were carried out with INANO, NANO, and CINANO at a 4:1 ratio of inhibitor to active sites in the preincubation mixture, no loss of activity was observed as expected for inhibition which is rapidly reversible. As a check, a similar experiment was performed with 3-FHB, a highly potent halohydroxybenzoate inhibitor whose inhibition is known to be freely reversible (May et al., 1978); similarly no inhibition was observed, as shown in Figure 2A. It is thus evident that the presence of a ketonizable 2-hydroxy group makes inhibition much less reversible, and a simple Michaelis-Menten treatment is insufficient to describe the interaction of HYPNO with PCD.

It is evident from Figure 1A that both the rate and extent of time dependent inactivation are dependent on HYPNO concentration with inactivation going to near completion at ratios of 20:1 and 40:1 HYPNO to enzyme active sites. However, at a 4:1 ratio inactivation does not go to completion, thus differing from the inactivation of HINANO which causes rapid complete inactivation even at a ratio of 1:1 HINANO to active sites (May et al., 1982). This implies that, in contrast to HINANO, HYPNO inactivation might be reversible by dialysis. Indeed, when an enzyme solution which had been inactivated by a 35:1 ratio of HYPNO to active sites was placed in an ultrafiltration cell and 10 volumes of buffer passed through, a regain of 47% of control activity was observed. Thus inactivation by HYPNO is intermediate between the kinetically irreversible inhibition caused by HINANO and the freely reversible binding of the halohydroxybenzoates. As expected, semi-log plots of the inactivation data (Figure 1B) therefore deviate from linearity in contrast to that for HINANO inactivation.

If it is assumed that the leveling in the 4:1 inactivation curve of Figure 1A reflects equilibration of slowly reversible HYPNO binding, an equilibrium constant can be calculated from the final extent of inactivation, assuming $K_D = \frac{[E]\,[HYPNO]}{[E \cdot HYPNO]}$. The results of such a calculation is a K_D of 4.2×10^{-6}M. A K_I for HYPNO inhibition was also obtained using the method developed by Henderson (1972), which has recently been applied to transition state analogs by Frieden et al.(1980). Assuming this treatment is valid for the slowly reversible HYPNO binding, and using the form of the Henderson equation appropriate for competitive inhibition, the K_I calculated from the slope of the Henderson plot is 1.6×10^{-6}M.

If the inactivation described above is due to interaction of HYPNO with the enzyme active site, then a freely reversible ground state analog such as 3-FHB should be capable of protecting against the inactivation due to HYPNO. As shown in Figure 2A, 3-FHB does indeed protect against inactivation. Moreover, the addition of 3-FHB to the HYPNO-inactivated PCD causes reactivation in a first order manner as shown in Figure 2A and 2B. The first order rate constant for the reactivation is 4.3×10^{-4} sec^{-1}.

Spectral Studies with HYPNO

The results discussed to this point establish that HYPNO binds to the active site of PCD in a slowly reversible manner. From the initial rates of the inactivation of Figure 1, a second order rate constant for HYPNO binding of 1×10^{2} M^{-1} sec^{-1} can be calculated which contrasts sharply with the k_{on} of 2×10^{6} M^{-1} sec^{-1} which we have previously reported for 3-FHB (May & Phillips 1979). We therefore proceeded to examine the spectral characteristics of HYPNO binding and to attempt to determine a k_{off} for the dissociation of HYPNO from PCD, in order to make a direct comparison with halohydroxybenzoate and HINANO binding.

When HYPNO at a 10 fold excess over active sites is added to the enzyme, and a spectrum taken after 30 minutes, the visible absorbance maximum is shifted to 485 nm as shown in Figure 3A. The absorbance change at 437 nm was followed and second order rate constants were calculated from the initial rates of change. These values, 130 M^{-1} sec^{-1} at 4:1 HYPNO: active sites, 120 M^{-1} sec^{-1} at 10:1, and 76 M^{-1} sec^{-1} at 20:1 give an average value of 1.1×10^{2} M^{-1} sec^{-1} which is in good agreement with the second order rate constant of 1×10^{2} M^{-1} sec^{-1} calculated from the inactivation data of Figure 1. Thus, we conclude that both the spectral change and the inactivation reflect the same process.

The determination of k_{off} for the PCD·HYPNO complex was attempted by displacement with an excess of the inhibitor, 3-FHB. However, as can be seen in Figure 3A, the changes are more complex than those expected for a simple displacement (Gutfreund, 1972). Addition of a 25 fold excess of 3-FHB to the equilibrated PCD·HYPNO solution shows little immediate change in the 420 nm region but a rapid significant decrease in absorbance at longer wavelengths. Later scans show a slow increase in the 420 nm region, with the final spectrum resembling that characteristic of the PCD·3-FHB complex (May et al., 1978). As shown in Figure 3B, when this slow spectral change is followed at 425 nm, a linear first order plot is obtained. The first order rate constant obtained from this plot is 2.4×10^{-4} sec^{-1} which is similar to the first order rate constant for the reactivation of PCD·HYPNO by the addition of 3-FHB (Figure 2) obtained under similar conditions. The rapid long wavelength change was also examined using stopped-flow techniques. When this rapid phase was examined at 570 nm under conditions of 2.5:1 3-FHB to HYPNO, a first order plot is obtained as shown in Figure 4. Under these conditions the rapid change is essentially complete in 15 sec. The first order rate constant calculated from the data in Figure 4 is 0.2 sec^{-1}.

The effects of 3-ClHB, a halohydroxybenzoate inhibitor which is somewhat less potent than 3-FHB (May & Phillips, 1979), on the PCD·HYPNO complex were also examined. As can be seen in Figure 5A, there is a similar rapid spectral change at long wavelengths followed by a slow increase in the 420 nm region. In the case of 3-ClHB the distinction between the rapid and slow processes is much clearer, since at long wavelengths the rapid process is characterized by a decrease in absorbance, and the slow process is characterized by an increase. The slow change followed at 420 nm gives a linear first order

plot (Figure 5B) with a first order rate constant of 1.5×10^{-4} sec^{-1}, similar to that obtained with 3-FHB. It can also be seen that the final spectrum indicates that the enzyme is not totally in the form of PCD·3-ClHB, as is evident from the spectrum of this complex shown in Figure 5A.

Taken together, the data obtained with both 3-FHB and 3-ClHB indicate that two distinct events occur when these hydroxybenzoate inhibitors interact with the preformed PCD·HYPNO complex. A very rapid spectral change is observed first at long wavelengths, followed by a slower process characterized by changes in the 420 nm region at a rate similar to that observed in the reactivation experiments. There are two obvious possibilities to explain these observations. The first requires the following assumptions: HYPNO binds to PCD causing the enzyme to adopt an altered conformation, PCD′. HYPNO can then rapidly dissociate from PCD′, and halohydroxybenzoate can also bind rapidly to this conformation, thus accounting for the rapid spectral change observed. Subsequently, the PCD′ halohydroxybenzoate complex undergoes a slow conformational change to give the normal ground state complex with normal spectral properties. The second possibility, which may also involve conformationally altered states of PCD, differs from the above in that it allows formation of a ternary complex. Halohydroxybenzoate inhibitor would thus bind rapidly to PCD·HYPNO to give this ternary complex, thus accounting for the rapid spectral change. Subsequent slow release of HYPNO would lead to formation of the normal PCD·3-FHB complex and recovery of activity. Therefore, dialysis experiments were carried out to distinguish between these possibilities.

Dialysis Experiments

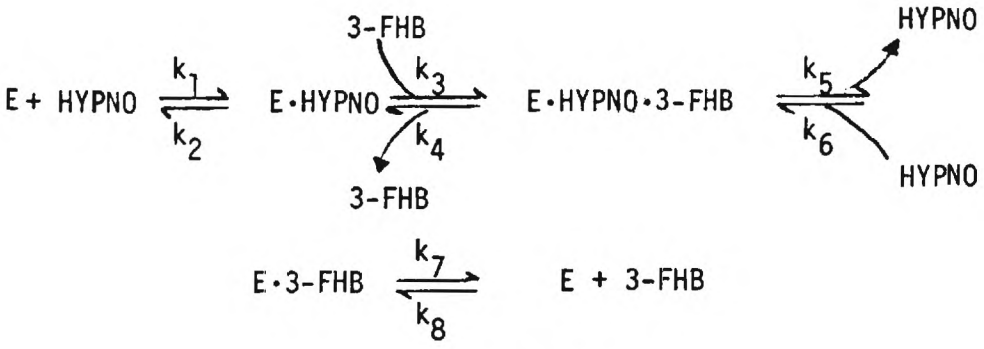
The two possibilities just mentioned to account for the spectral data differ in that the first implies very rapid release of HYPNO and binding of halohydroxybenzoate; whereas, the second possibility implies a much slower release of HYPNO with a half-life of approximately 50 minutes. Thus, these two possibilities should be clearly distinguishable in dialysis experiments

designed to determine which species are bound to the enzyme immediately after addition of the halohydroxybenzoate to the enzyme·HYPNO complex.

A solution containing equimolar HYPNO and enzyme active sites was allowed to come to equilibrium, either 3-FHB or 3-ClHB was added, and the solution was immediately subjected to ultrafiltration. The concentrations of free HYPNO and halohydroxybenzoate in the effluent were then determined spectrophotometrically. The results obtained under various experimental conditions are shown in Table II. It should be noted that approximately 3-4 minutes were required to obtain effluents in these experiments, which corresponds closely to the time elapsed at the end of the spectral scans designated with triangles in Figures 3A and 5A. Four primary observations can be made from the data in Table II.

1. Stoichiometric binding of 3-FHB to the enzyme is observed with a 2.5 fold excess of 3-FHB.
2. Greater than 1:1 binding of total inhibitor (halohydroxybenzoate + HYPNO) to active sites is always observed.
3. The amount of HYPNO bound to enzyme is unaffected by the quantity of 3-FHB bound.
4. The amount of HYPNO bound to PCD is unaffected by the use of different halohydroxybenzoate inhibitors.

Taken together, these results are consistent with formation of a ternary complex, and a minimal scheme incorporating such a complex is shown in Scheme II.



SCHEME II

Direct support for the postulated ternary complex is provided by the results of the dialysis experiments which established greater than stoichiometric binding of total inhibitor to the enzyme. Moreover, it is obvious from the spectral studies that the rapid process leads to formation of some complex which is spectrally distinct from either the enzyme·HYPNO complex or the normal enzyme·halohydroxybenzoate complex. This is accounted for by rapid formation of the ternary E·HYPNO·3-FHB complex. On the other hand, the slow process seen in the spectral studies represents dissociation of HYPNO from the ternary complex to form E·3-FHB, and this is fully consistent with the slow appearance of the characteristic E·halohydroxybenzoate complex.

Assuming this minimal scheme, values can be assigned to the various rate constants from the results of our spectral and inhibition experiments, and these values are listed in Table III. The value for k_1 is obtained from the inactivation data in excellent agreement with that obtained from direct spectrophotometric measurement of HYPNO binding. The value of $K_D^{(1)}$, the dissociation constant for HYPNO binding given by k_2/k_1, is obtained from the extent of HYPNO inactivation at equilibrium (Figure 1). It is interesting that this value agrees well with the kinetically determined K_I^{HYPNO} obtained using the Henderson analysis, but differs markedly from the value obtained from a simple Michaelis-Menten treatment. With these values in hand, the magnitude of k_2 is calculated directly as approximately 3×10^{-4} sec^{-1}, which is in accord with our expectation that HYPNO dissociation should be a slow process. Values for k_3 and k_5 are obtained from the rates of the rapid and slow spectral changes, respectively (Figures 3 and 4). An internal check on the value of k_5 is provided by the 3-FHB reactivation data (Figure 2). Under the conditions of this experiment, the magnitude of k_3 predicts that

the ternary complex should be formed rapidly and the reactivation process actually reflects k_5. There is, in fact, good agreement between the values of k_5 obtained from these two sets of experiments. The values listed for k_7 and k_8 are taken from our previous results with halohydroxybenzoate inhibitors (May & Phillips, 1979).

The results of the dialysis experiments provide a value for $K_D^{(2)}$, the dissociation constant for 3-FHB release from the ternary complex, since under the conditions of these experiments, ternary complex formation is essentially complete, and the extent of HYPNO dissociation (k_5) is negligible. The concentrations of free 3-FHB and HYPNO therefore provide a measure of the magnitude of $K_D^{(2)}$, calculated as described in the Methods Section. This value is found to be 16×10^{-6}M and a similar calculation from the data for 3-ClHB provides a value of 48×10^{-6}M for $K_D^{(2)}$ of this compound.

Further support for Scheme II is provided by the following: The spectral studies predict that under the conditions of the dialysis experiments the fast process should be essentially complete and the slow process should not have begun. This is supported by the data in Table II which shows that the amount of HYPNO bound to the enzyme is not affected by either the identity or quantity of the halohydroxybenzoate inhibitor. Therefore, the amount of HYPNO bound reflects only the equilibrium position of the initial binding process, and a value of $K_D^{(1)}$ should thus be obtainable from the measured amount of free HYPNO and the known initial concentrations. According to this reasoning, the value of $K_D^{(1)}$ calculated from these data should agree with that determined independently from the extent of inactivation at equilibrium (Figure 2). As shown in Table III this is, in fact, the case. It is important to note that the value obtained from $K_D^{(1)}$ in this manner is independent of whether 3-FHB or 3-ClHB is used in the dialysis experiments, and this provides strong support for interpretation of our data in terms of Scheme II. However, it should be noted that this is a *minimal* scheme

which does not distinguish between different conformational states of PCD.

Reactivation by Dilution

Since the rate of dissociation of HYPNO from PCD·HYPNO could not be determined by displacement because of the formation of a ternary complex, reactivation by dilution was carried out. Schloss and Cleland (1982) have recently used this method to determine the upper limit of a dissociation rate constant for isocitrate lyase complexed to the reaction-intermediate analog, 3-nitropropionate. When a concentrated enzyme·HYPNO solution is diluted 1000 fold into an assay solution containing 400 μm PCA a slow increase in activity is seen, as shown in Figure 6. The final maximum rate is extrapolated back to the x-axis. The reciprocal of the x-axis value at the extrapolated line intersection is equal to k, the dissocation rate constant, if reactivation goes to completion. Since the final rate is only 53% of the uninhibited rate due to substrate depletion, the value obtained, $k_2 = 13.7 \times 10^{-4}$ sec^{-1}, is an upper limit for the dissociation rate constant. This value is clearly consistent with the calculated values for k_2 in Table III.

Inactivation by MINANO

The data in Table I establishes that various pyridine N-oxides are capable of interacting with protocatechuate 3,4-dioxygenase, but as discussed above, the presence of a ketonizable 2-hydroxy group in HYPNO and HINANO results in inhibition which is not freely reversible. We were therefore interested in examining the affect of a 2-sulfhydryl substituent on pyridine N-oxide inhibition, since this functionality would also be expected to exist primarily in the keto form (Jones & Katritzky, 1960). As shown in Figure 7, incubation of the enzyme with MINANO followed by dilution into an excess of assay solution results in time dependent inhibition of the type observed previously for HYPNO (Figure 1A).

Since rapidly reversible inhibition would not be seen under these conditions, it is evident that the presence of a 2-thio substituent does indeed interefere with reversibility. The data in Figure 7 indicate that both the rate and extent of inhibition are dependent on MINANO concentration, with inhibition going to completion at a ratio of 4:1 MINANO to active sites. This contrasts with the oxygenated analog, HINANO, where inhibition goes rapidly to completion at ratios of 1:1 (May et al.,1982). As expected, MINANO is a considerably more potent inhibitor than HYPNO, since the former possesses a carboxyl moiety which presumably interacts at the cationic site which binds the carboxyl of the normal substrate. However, it is clear that although the carboxyl affects inhibitor potency it is the presence or absence of a ketonizable substituent in the 2-position which determines whether or not inhibition will be freely reversible.

DISCUSSION

The interaction of PCD with various halohydroxybenzoate ground state inhibitors has been previously studied in this and other laboratories (May et al., 1978; May & Phillips, 1979; Felton et al, 1978; Fujisawa et al, 1971; Fujisawa et al., 1972a;Fujisawa et al., 1972b; Que et al., 1977; Keyes et al., 1978; Tatsuno et al.,1978; Nakata et al.,1978). For example, we have reported that the most potent of the halohydroxybenzoate inhibitors, 3-FHB, binds stoichiometrically to the enzyme; however, its inhibition is rapidly reversible and is not apparent when the complex is diluted into a large excess of substrate. The halohydroxybenzoates, 3-FHB and 3-ClHB, also give a spectral change upon binding to PCD which is characterized by a shift of the visible maximum from 450 to 420 nm. This characteristic shift occurs under both aerobic and oxygen deficient conditions (May et al.,1978).

The behavior of the halohydroxybenzoates contrasts with that of HINANO, a compound designed to mimic the ketonized form of the PCD substrate, PCA. As we have previously reported (May et al., 1982), HINANO binds stoichiometrically to the active site of PCD but does so in an essentially irreversible manner, being released only after denaturation of the enzyme. HINANO is an excellent active site titrant and is capable of completely displacing the most potent halohydroxybenzoate, 3-FHB, from the active site; whereas, even an excess of 3-FHB does not displace HINANO. The PCD·HINANO complex exhibits spectral properties resembling those of an advanced intermediate along the substrate reaction pathway (Bull et al., 1981). However, due to the irreversibility of HINANO binding, a detailed kinetic study using standard techniques was not feasible.

The data presented in this paper establish that HYPNO, which has the same 2-hydroxypyridine N-oxide structure as HINANO but lacks the carboxyl, is also not a freely reversible inhibitor, thus differing from the halohydroxybenzoates. However, we have found that HYPNO inhibition, unlike that of HINANO ,is slowly reversible, thus allowing a complete kinetic characterization of its interaction with PCD. Our results allow a direct comparison of the rate constants

for binding and dissociation of HYPNO with those for 3-FHB. The k_{on} for HYPNO binding to PCD is 10^2 M^{-1} sec^{-1} whereas the k_{on} for 3-FHB is 2×10^6 M^{-1} sec^{-1} (May & Phillips, 1979). Likewise, the k_{off} for HYPNO is approximately 3×10^{-4} sec^{-1} and the k_{off} for 3-FHB is approximately 1 sec^{-1}. Thus, both binding and dissociation rate constants are four orders of magnitude less for HYPNO. Although the K_D value for 3-FHB (0.5×10^{-6}M) is less than that for HYPNO (3×10^{-6}M) implying comparable binding of the former, the low values of the on and off rate constants for HYPNO are responsible for this compound exhibiting time dependent inactivation which is only slowly reversed by dilution or dialysis.

It is clear from studies with other enzymes, such as adenosine deaminase and adenylate deaminase, that transition state analogs characteristically appear to have "on" rate constants which are considerably smaller than those expected for diffusion-controll processes (Frieden et al., 1980; Wolfenden, 1976; Kurz & Frieden, 1983). For example, the k_{on} values for various coformycin analogs to adenylate deaminase are all on the order of 10^4 M^{-1} sec^{-1}. These values are quite similar to those which we have previously reported for HINANO binding to PCD (May et al., 1982). By comparison, the k_{on} for HYPNO is about 2 orders of magnitude slower. This is not unexpected, since HYPNO lacks the carboxylate functionality which is known to increase binding potency, and this could be at least partially reflected in k_{on}. The k_{off} for HYPNO is also very similar to the values reported for the coformycin analogs. Therefore, it seems clear that the presence of the ketonizable 2-hydroxy group and the hydroxamic acid-like p-oxygen which is an excellent iron ligand (Neilands, 1966; Chatterjee, 1978), are sufficient to confer the "transition-state analog-like" characteristics of slow binding and dissociation on PCD inhibitors. The other pyridine N-oxides studied (Table I) which lack these features do not exhibit these characteristics. On the other hand, MINANO with a ketonizable 2-thiol substituent, does exhibit the characteristics of HINANO and HYPNO inhibition.

The results of our spectral and dialysis experiments on the interactions of halohydroxybenzoates with the E·HYPNO complex are consistent with formation of a ternary E·HYPNO·3-XHB complex. There is no precedent for formation of such a ternary complex in all previous studies with PCD inhibitors and substrate analogs, and thus this result was unexpected. On the basis of information in the literature regarding binding interactions at the PCD active site, it is tempting to speculate about a possible physical explanation for this result. One can visualize that in the case of substrate and halohydroxybenzoate inhibitors since binding involves both ligation of the para hydroxyl and ionic interaction of the carboxylate with a cationic group of the enzyme--likely an arginine -- (May & Phillips, 1979; Carlson et al., 1980), the active site is pinned into a closed conformation. An additional substrate or inhibitor molecule cannot be accommodated in this conformation and ternary complexes do not occur. However, upon binding of HYPNO which contains a potent iron-ligating functionality but no carboxyl, the cationic site may remain sufficiently accessible to interact with the carboxylate of a 3-XHB molecule without immediate ligation of its para hydroxyl.

It should be noted that Scheme II represents a *minimal* scheme which should be regarded at this point as a working hypothesis on which to base further experiments. As indicated in Table III, assumption of this scheme results in very good internal consistency between the various rate and equilibrium constants obtained from inactivation, spectral, and dialysis experiments. However, different conformational states of PCD are not taken into account and the values for several of the rate constants have not been independently confirmed. Thus, the actual detailed pathway for formation and decomposition of the postulated ternary complex could be considerably more complex.

Previous resonance Raman investigations in this laboratory (Felton et al.,1978) and by Que and Epstein (1981) have provided evidence for the direct ligation of the para hydroxyl of hydroxybenzoate inhibitors to iron at the active site of PCD. These studies have also shown the presence of two tyrosine ligands which are not displaced upon substrate or inhibitor binding. Recent studies from our laboratory using resonance Raman and extended X-ray absorption fine structure (EXAFS) have provided further insight into the ligation environment at the site of PCD (Felton et al.,1982). These studies have provided evidence for the presence of two histidine ligands, one of which is displaced upon binding of the halohydroxybenzoate, 3-ClHB. Thus, as has been previously suggested, histidine may become available to act as a general base, providing for the removal of even relatively nonacidic p-hydroxyl protons (such as those found in all PCD substrates), as the hydroxyl nears the coordination sphere of iron. In view of the spectral differences seen between the halo-hydroxybenzoate and HINANO or HYPNO complexes with PCD, it will be interesting to see whether EXAFS studies are capable of revealing ligation differences between these various complexes. Further studies along these lines are in progress.

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TABLE I

Compound	Apparent K_I
HINANO	6.8μM
INANO	1.3 mM
HYPNO	3.9 mM
CINANO	21 mM
NANO	35 mM

TABLE II

ENZYME INHIBITOR DIALYSIS EXPERIMENTS[a]

Halohydroxybenzoate Inhibitor	Ratio of Halohydroxybenzoate : HYPNO:PCD Active Sites	
	Initial [b]	Bound
3-FHB	2.5:1 : 1	
	Expt. 1	1.1:0.70:1
	Expt. 2	0.89:0.64:1
	1:1:1	
	Expt. 1	0.58:0.73:1
	Expt. 2	0.59:0.66:1
C-ClHB	1:1:1	0.43:0.74:1

[a]HYPNO was incubated with the enzyme for 30 minutes at room temperature followed by addition of 3-FHB or 3-ClHB and immediate ultrafiltration. The filtration was complete 3-4 minutes after the addition of 3-FHB or 3-ClHB. See Materials and Methods for details.

[b]The actual initial concentrations for 3-FHB, 2.5:1:1 were Expt. 1: [HYPNO]=[enzyme active sites] = 24.2×10^{-6} M and [3-FHB] = 60.4×10^{-6} M, Expt. 2: [HYPNO] = [enzyme active sites] = 26.4×10^{-6} M and [3-FHB] = 65.8×10^{-6} M; for 3-FHB, 1:1:1, Expt.1: [3-FHB] = [HYPNO] = [enzyme active sites] = 26.6×10^{-6} M, Expt. 2: [3-FHB] = [HYPNO] = [enzyme active sites] = 25.6×10^{-6} M; and for 3-ClHB, 1:1:1, [3-ClHB] = [HYPNO] = [enzyme active sites] = 26.6×10^{-6} M.

TABLE III

Process	Rate Constants		Equilibrium Constants		
HYPNO Binding to Enzyme	k_1	1×10^2 M^{-1} sec^{-1} [a]	K_I	1.6×10^{-6} M	j
		1.1×10^2 M^{-1} sec^{-1} [b]	K_D (1)	4.2×10^{-6} M	k
	k_2	1.6×10^{-4} sec^{-1} c		2.6×10^{-6} M	l
		4.2×10^{-4} sec^{-1} d		4.3×10^{-6} M	l
				2.3×10^{-6} M	m
3-FHB Binding to E ·HYPNO	k_3	300 M^{-1} sec^{-1} e	K_D (2)	16×10^{-6} M	n
	k_4	4.8×10^{-3} sec^{-1} f			
HYPNO Release from Ternary Complex	k_5	4.3×10^{-4} sec^{-1} g			
		2.4×10^{-4} sec^{-1} h			
3-FHE Binding to Enzyme	k_7	1 sec^{-1} i	K_I	0.5×10^{-6} M	i
	k_8	2×10^6 M^{-1} sec^{-1} i			

a Calculated from initial rates of inactivation

b Calculated from initial rates of spectral change

c Calculated from k_1 K_I

d Calculated from k_1 times the K_D value from equilibrium inactivation data

e Calculated from results of the stopped-flow study of the fast spectral change

f Calculated from k_3 K_D

g Calculated 3-FHE reactivation data

Table III (footnotes continued)

h Calculated from the slow spectral change initiated by 3-FHB addition

i See (May & Phillips, 1979).

j Calculated from the Henderson Plot

k Calculated from equilibrium inactivation data

l Calculated from the dialysis 1:1:1 experiments with 3-FHB

m Calculated from the 1:1:1 dialysis experiment with 3-ClHB

n Calculated from the 1:1:1 dialysis experiments with 3-FHB (average of 15.6×10^{-6} M and 16×10^{-6} M).

FIGURE 1

TIME DEPENDENT INACTIVATION OF ENZYME BY HYPNO

(A) Time course of inactivation of the enzyme by HYPNO was followed by incubating at room temperature 0.5 ml of 0.331 μM enzyme (2.65 μM enzyme active sites) in 0.05 M Tris·HCl, pH 7.5 at 25°C, which was 10.6 μM (o), 53 μM (Δ), and 106 μM(□) in HYPNO giving ratios of 4:1 (o), 20:1 (Δ), and 40:1 (□) HYPNO : enzyme active sites. These solutions were assayed by adding 25 ul to 3 ml of an assay solution containing 300 ul of 0.5 M Tris·HCl, pH 7.5, 100 ul of 12 mM protocatechuic acid and 2.6 ml of H_2O and following the A_{290} at 25°C with a DW-2 spectrophotometer (Aminco). Initial rates of decrease in [protocatechuic acid] were calculated using $\varepsilon = 2280\ M^{-1}\ cm^{-1}$ and compared to a control solution containing no HYPNO which retained full activity throughout the inactivation period. (B) First order plot of the data in A. Second order rate constants calculated from initial rates of inactivation are 93.1 $M^{-1}\ sec^{-1}$ (o), 99.1 $M^{-1}\ sec^{-1}$ (Δ) and 121 $M^{-1}\ sec^{-1}$ (□).

FIGURE 2

3-FHB PROTECTS PCD AGAINST INACTIVATION BY HYPNO AND REACTIVATES HYPNO-INACTIVATED PCD

(A) Time course of inactivation of the enzyme was followed by incubating at room temperature 0.5 ml of 0.314 μM enzyme (2.51 μM enzyme active sites) in 0.05 M Tris·HCl, pH 7.5 at 25°C, which was 50.2 μM in 3-FHB (o), 50.2 μM in 3-FHB and 50.2 μM in HYPNO (Δ), or 50.2 μM in HYPNO followed by addition of 3-FHB to a concentration of 124 μM at arrow (□). These incubations gave ratios of 20:1 3-FHB: active sites (o), 20:20:1 3-FHB:HYPNO: active sites (Δ), and 20:1 HYPNO:active sites followed by 50:20:1 3-FHB: HYPNO:active sites at arrow. The assays were carried out as in Figure 1A. (B) First order plot of the reactivation in 2A assuming infinite reactivation to 77%. Times given are minutes following addition of 3-FHB.

FIGURE 3

SPECTRAL STUDY OF ENZYME, HYPNO AND 3-FHB INTERACTIONS

(A) Spectra were taken with a DW-2 spectrophotometer thermostated at 25°C. The enzyme solution was 11.6×10^{-6}M in active sites in 0.05M Tris·HCl, pH 7.5 at 25°C, (——). This solution was preincubated for 30 min with HYPNO at a ratio of 10:1 HYPNO:active sites and scanned (---). The inhibitor 3-FHB was then added to give a ratio of 25:1 3-FHB:HYPNO and this solution was scanned immediately (- Δ -Δ), after 1.5 hours (-o-o) and after 3.5 hours (-□ -□). Intermediate scans were also made but omitted for the sake of clarity. A separate solution containing the same concentrations of only enzyme and 3-FHB was scanned after 3.5 hours of preincubation (x x x x).

(B) Values of $A_\infty - A_t$ at 425 nm were calculated assuming $A_\infty = 0.0569$ for all scans of the solution containing enzyme, HYPNO, and 3-FHB. These values give a linear semilogarithmic plot.

FIGURE 4

KINETICS OF THE FAST SPECTRAL CHANGE

A solution in 0.05 M Tris·HCl, pH 7.5 at 25°C containing 26.6 x 10^{-6} M, enzyme active sites and 26.6 x 10^{-5}M HYPNO was preincubated for at least 30 min before flowing against a 665 x 10^{-6}M 3-FHB solution in the same buffer (final concentrations are one-half those given). The experiment was carried out in a DW-2 spectrophotometer (Aminco) and the fast change was followed at 570 nm with the reference at 750 nm.

FIGURE 5

SPECTRAL STUDY OF ENZYME, HYPNO AND 3-ClHB INTERACTIONS

(A) This experiment was performed exactly as in Figure 3 except 3-ClHB was used in place of 3-FHB. The enzyme solution was scanned alone (——), after 30 min preincubation with HYPNO (- - -), immediately after the addition of 3-ClHB (- Δ- Δ), 2 hours later (-o-o), and 4.5 hours later (- □- □). Intermediate scans are again omitted for clarity. A separate solution containing the same concentrations of only enzyme and 3-ClHB was scanned after 4.5 hours of preincubation (x x x x). (B) Values of $A_\infty - A_t$ at 420 nm were calculated assuming $A_\infty = 0.057$ for all scans of the solution containing enzyme, HYPNO, and 3-ClHB. These values give a linear semilogarithmic plot.

FIGURE 6

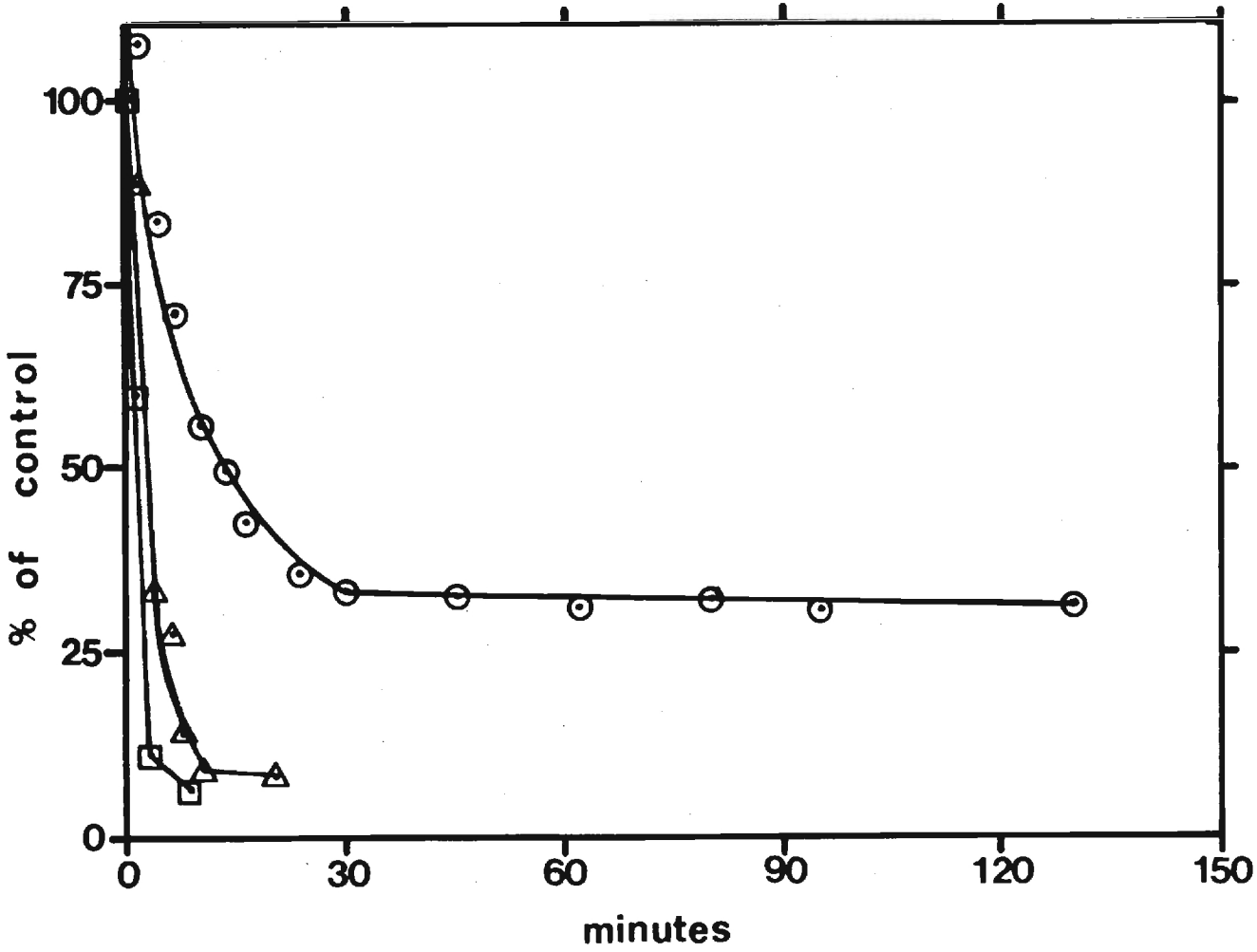
REACTIVATION OF ENZYME·HYPNO COMPLEX BY DILUTION

A solution of 5.3×10^{-6}M enzyme active sites in 0.05M Tris·HCl, pH 7.5 at 25°C was incubated with 1.06×10^{-4}M HYPNO (20 fold excess) for 30 min. Three microliters of this solution were then added to 3 ml of an assay solution containing 400×10^{-6}M PCA and 0.05 M Tris·HCl, pH 7.5 at 25°C and the A_{290} was followed in a Varian DMS 90 spectrophotometer. A control solution the same as above, but without HYPNO, was assayed in the same manner. Both assays were followed to the extent of substrate depletion which began to diminish the reaction rate of the control.

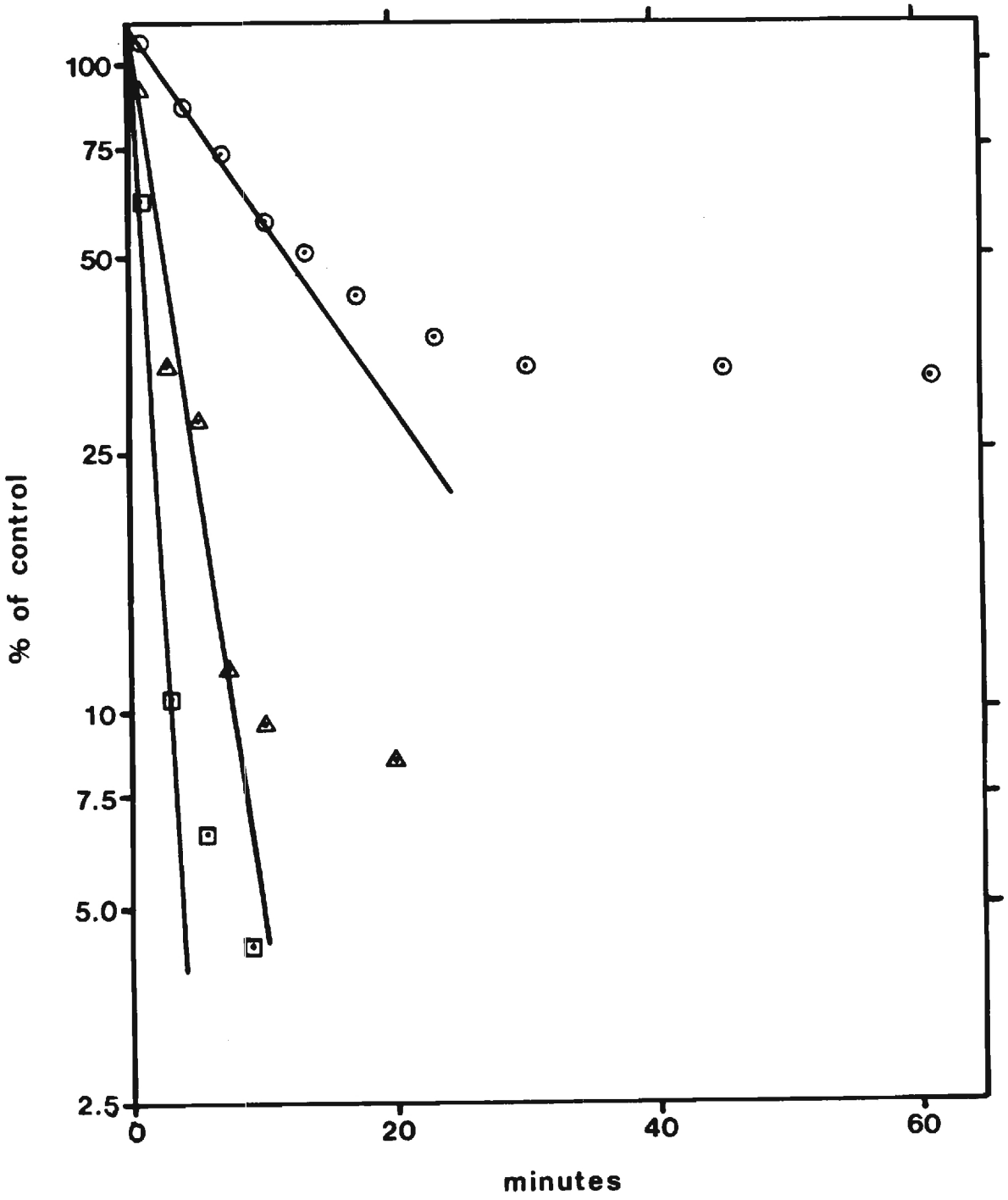
FIGURE 7

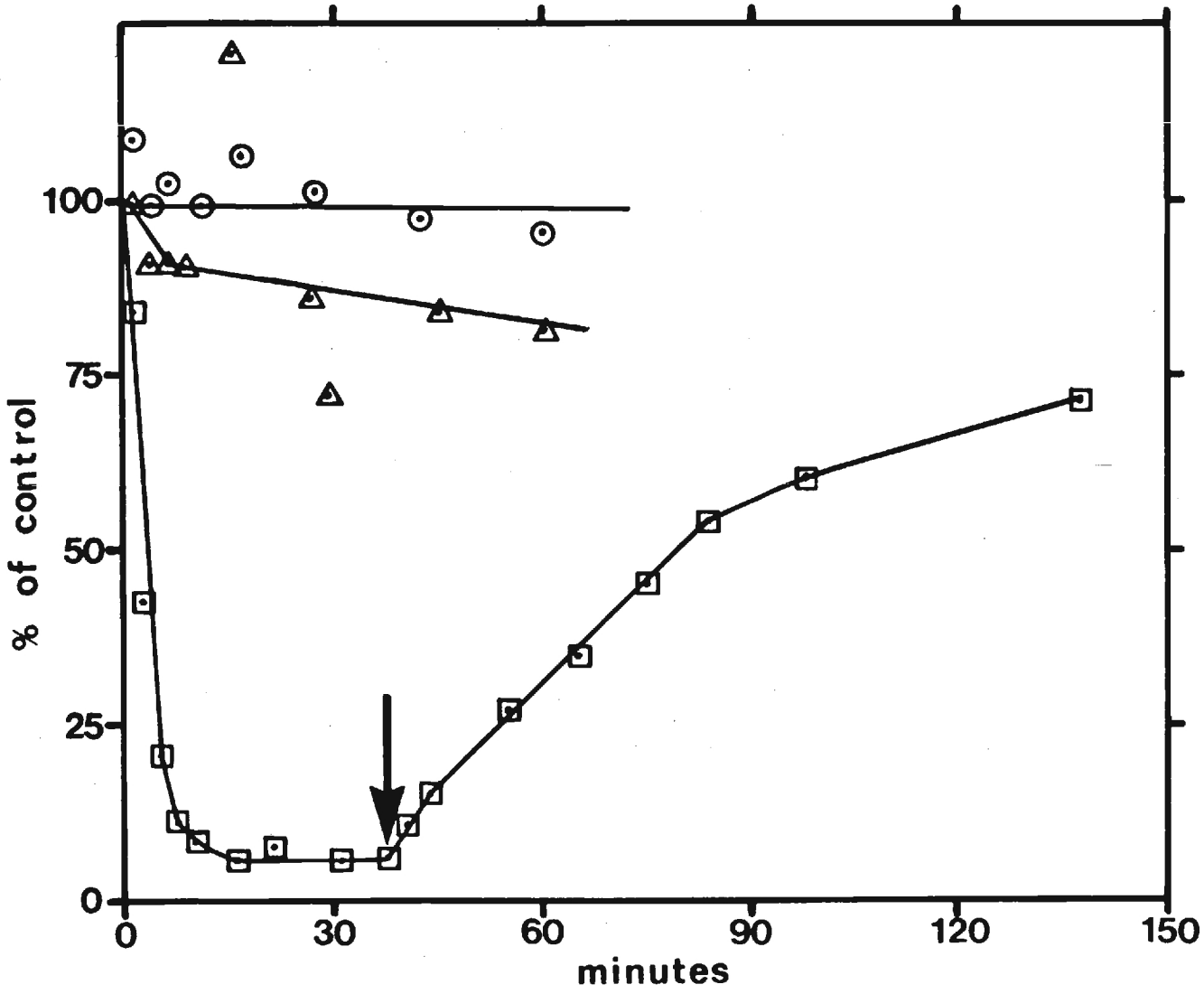
TIME DEPENDENT INACTIVATION OF ENZYME BY MINANO

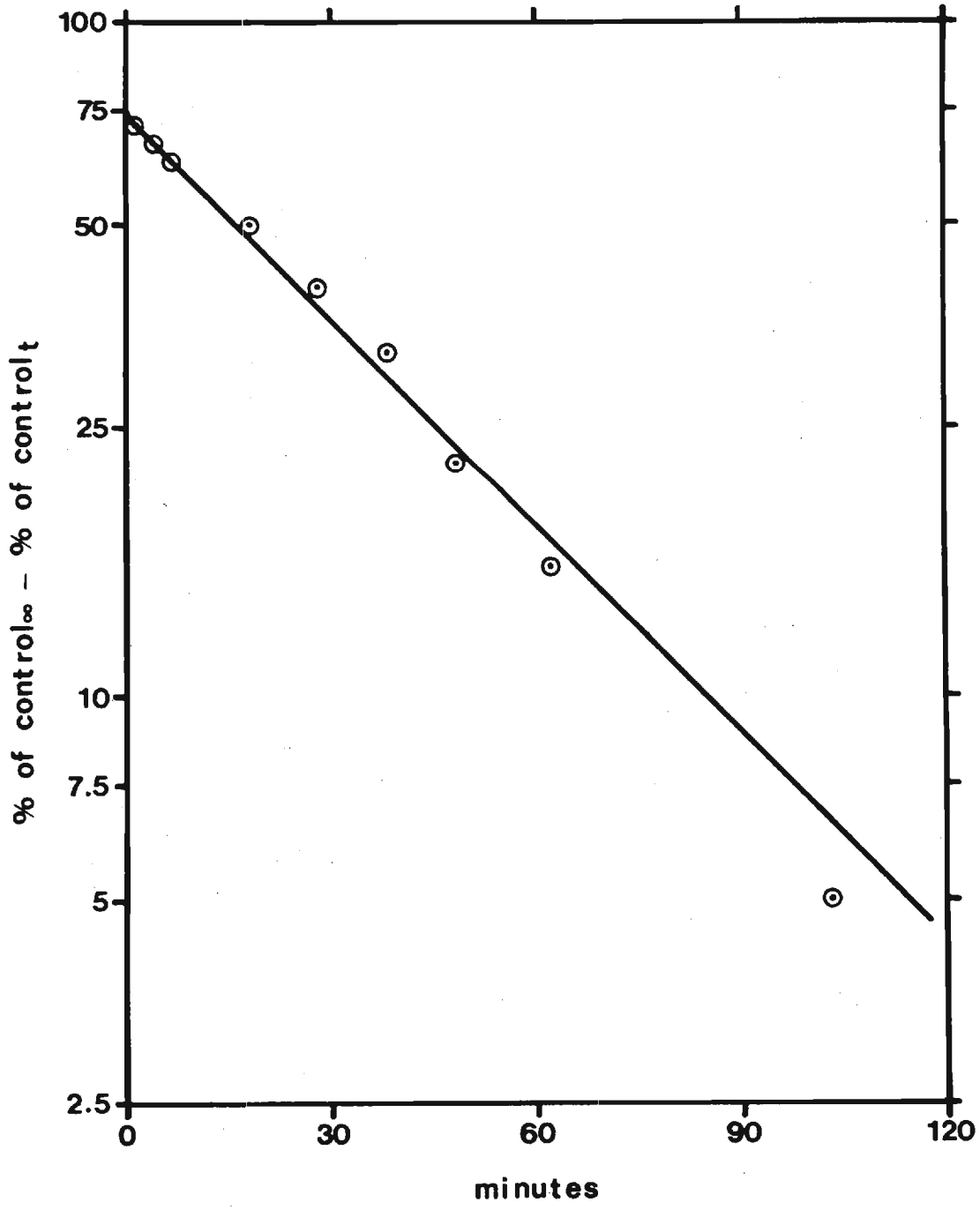
The time course of inactivation of enzyme by MINANO was followed by incubating at room temperature 1 ml of 2.5×10^{-6} to 4.0×10^{-6} M enzyme active sites in 0.05 M Tris·HCl, pH 7.5 at $25^{o}C$, which was 1:1 (o) 2:1 (△) and 4:1 (□) in the ratio of MINANO to enzyme active sites. These solutions were assayed as described in Figure 1 except 20 ul of the 4:1 solution was added to the assay mixture and the A_{290} was followed on a Varian DMS 90 spectrophotometer.

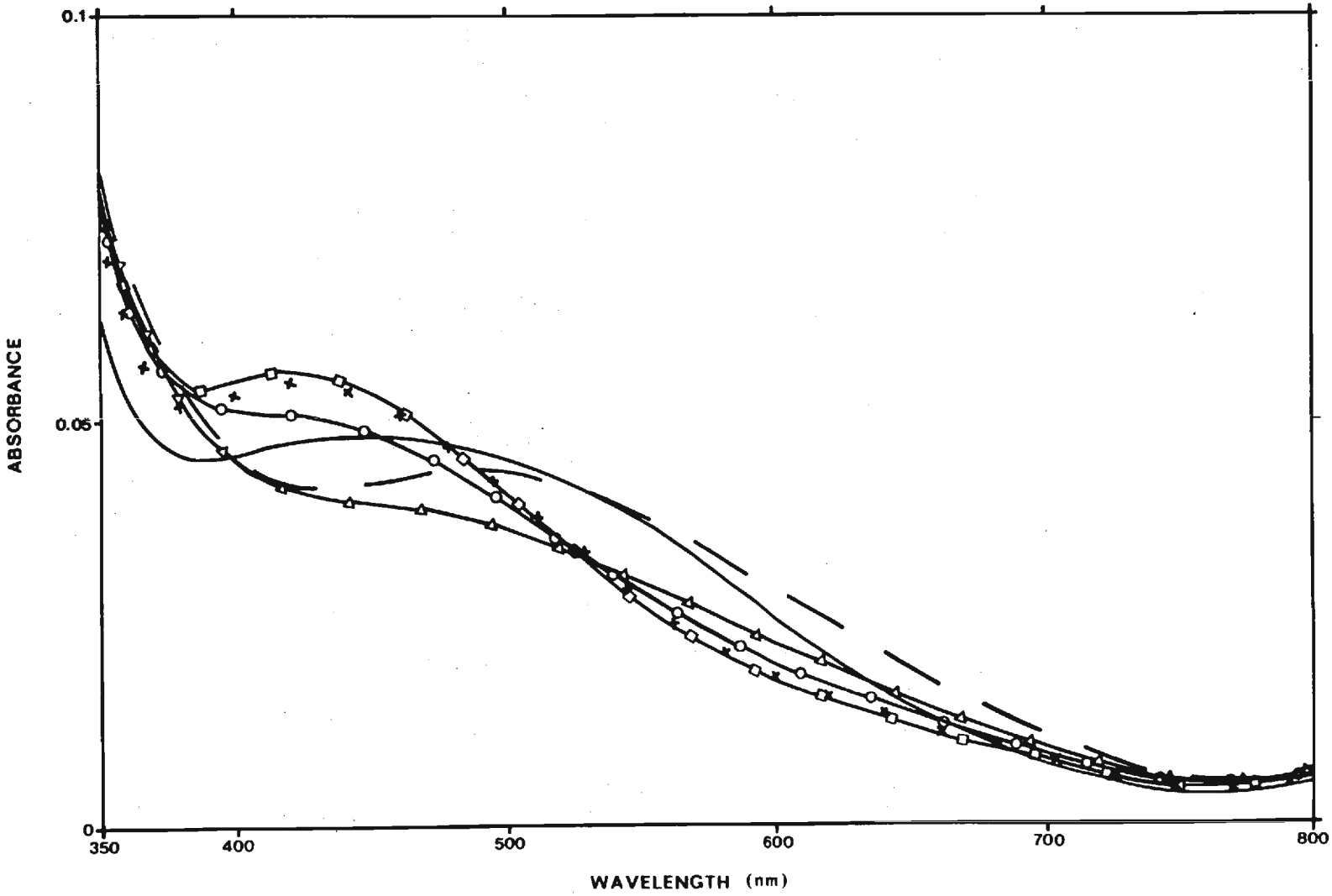


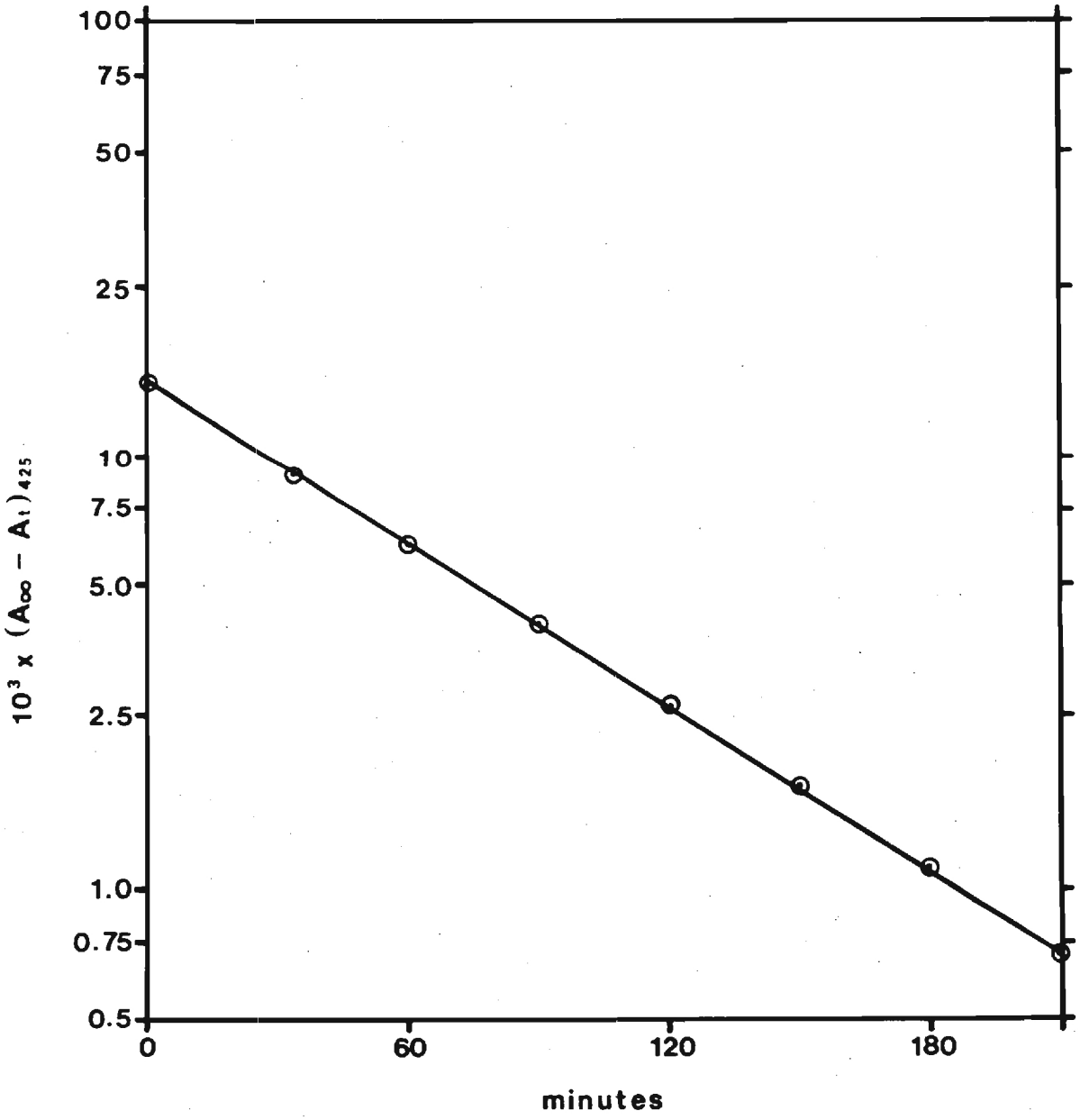
MAY/FELTON 320445583

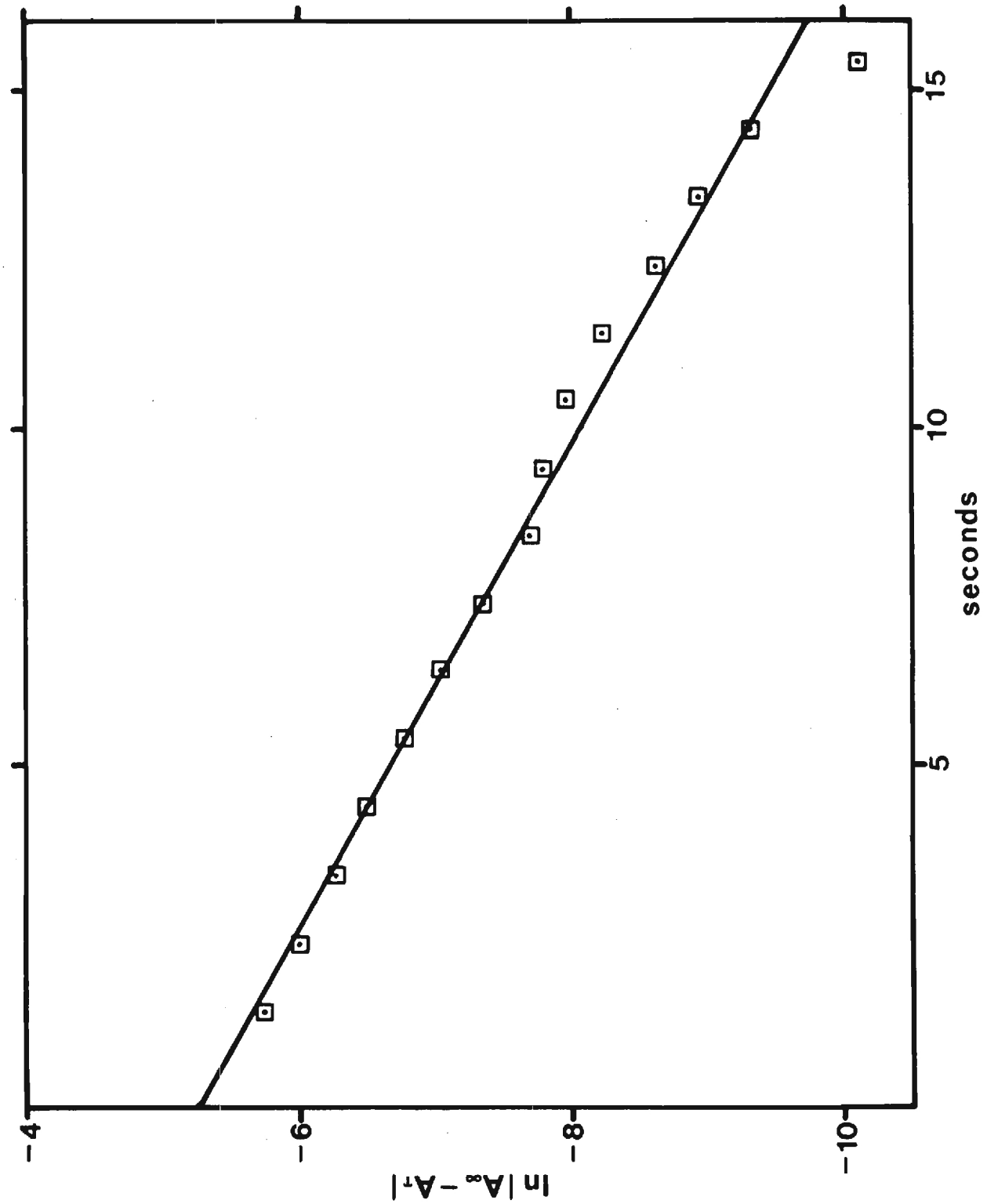

100
75
50
25
10
7.5
5.0
2.5
% of control
0
20
40
60
minutes

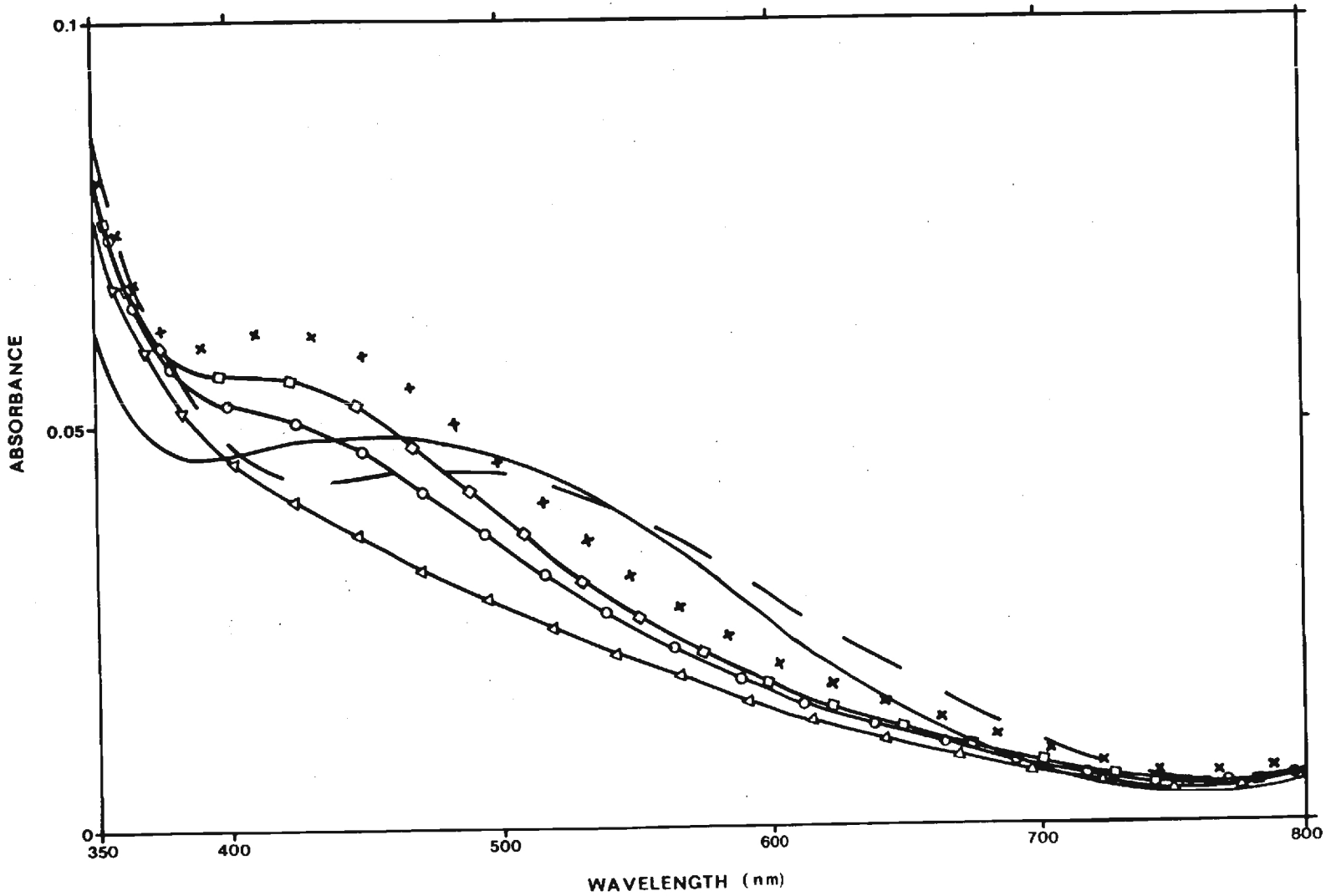


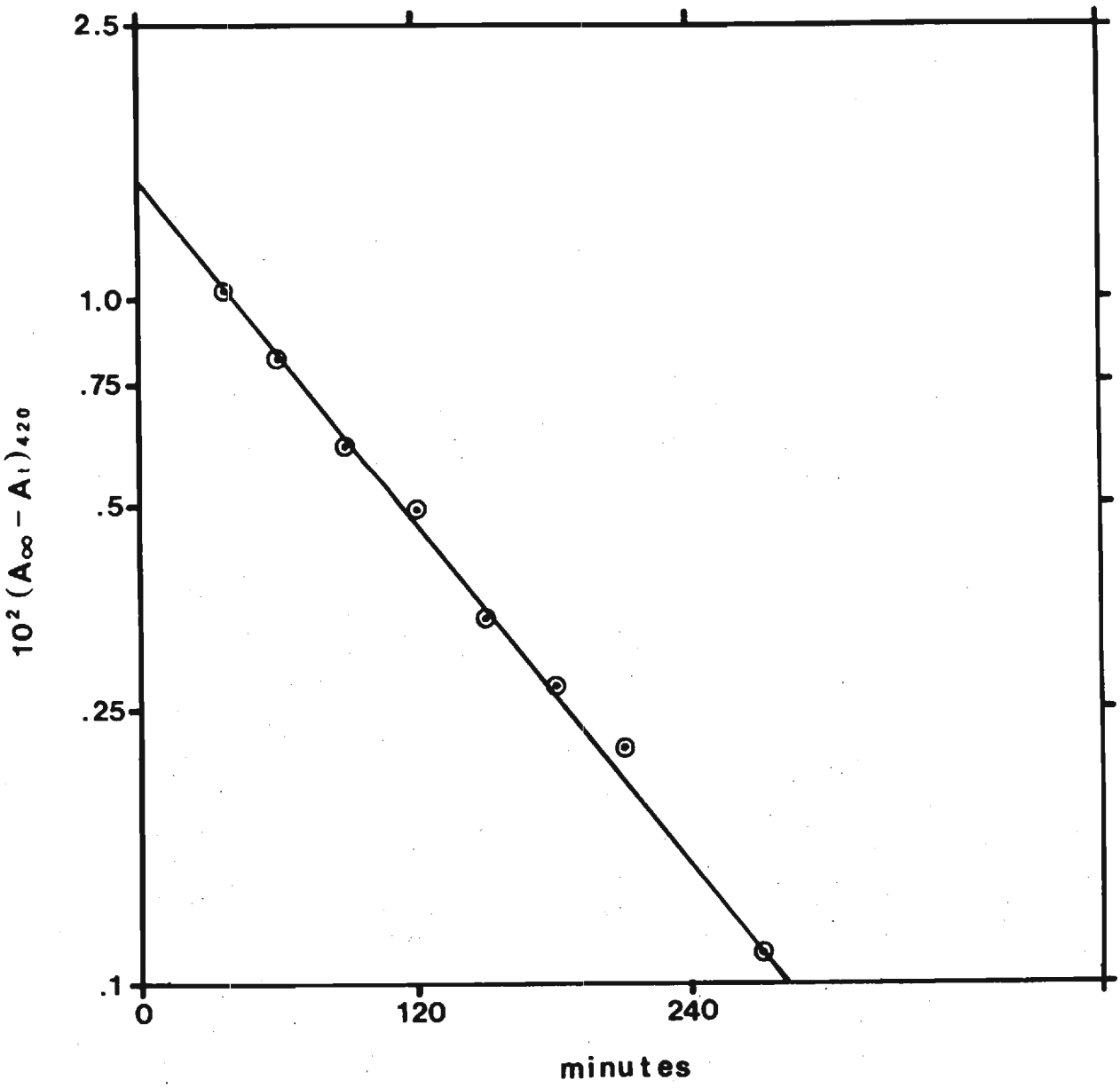

100
75
50
25
10
7.5
5
2.5
0
30
60
90
120
% of control∞ - % of controlt
minutes

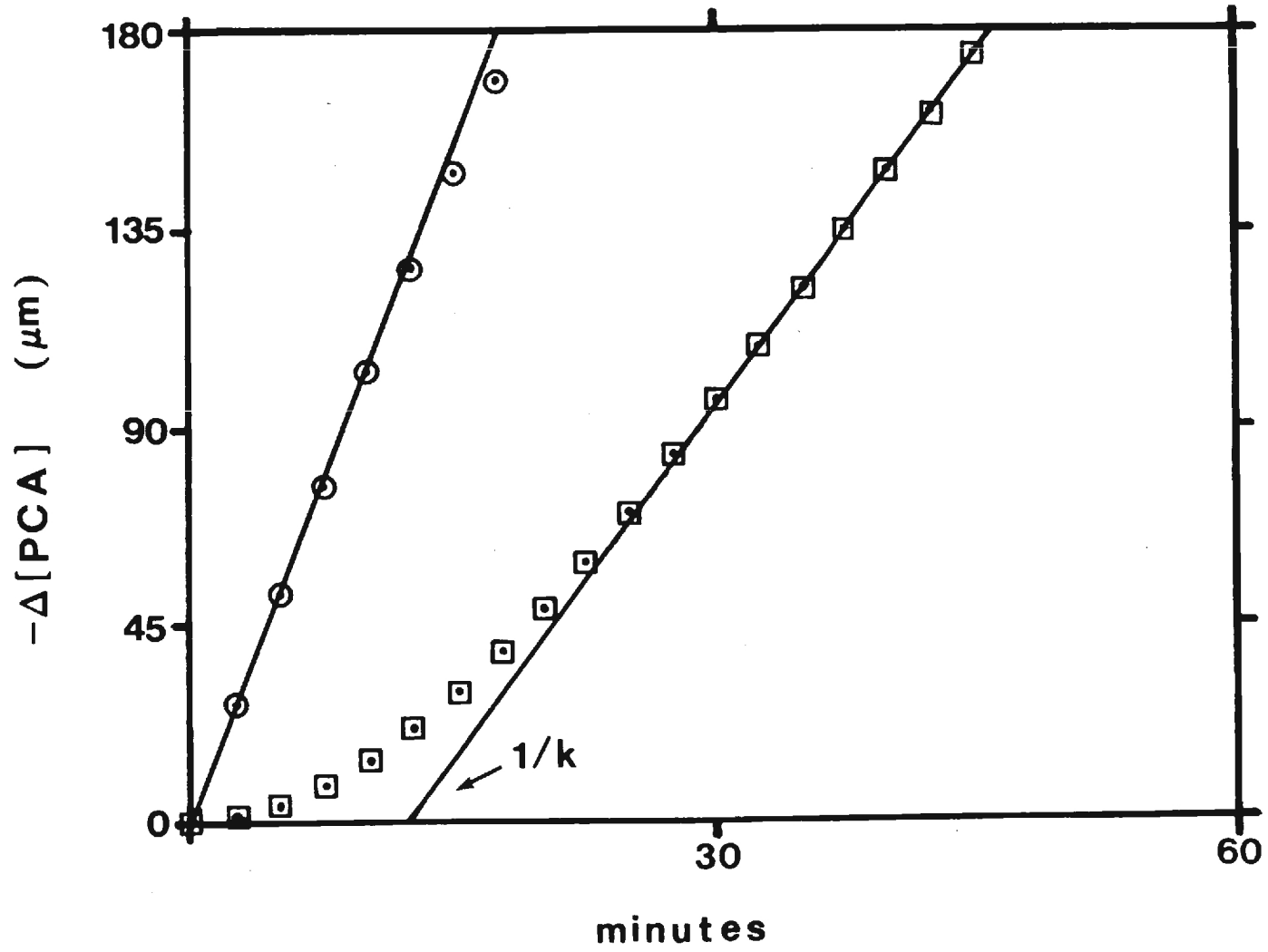


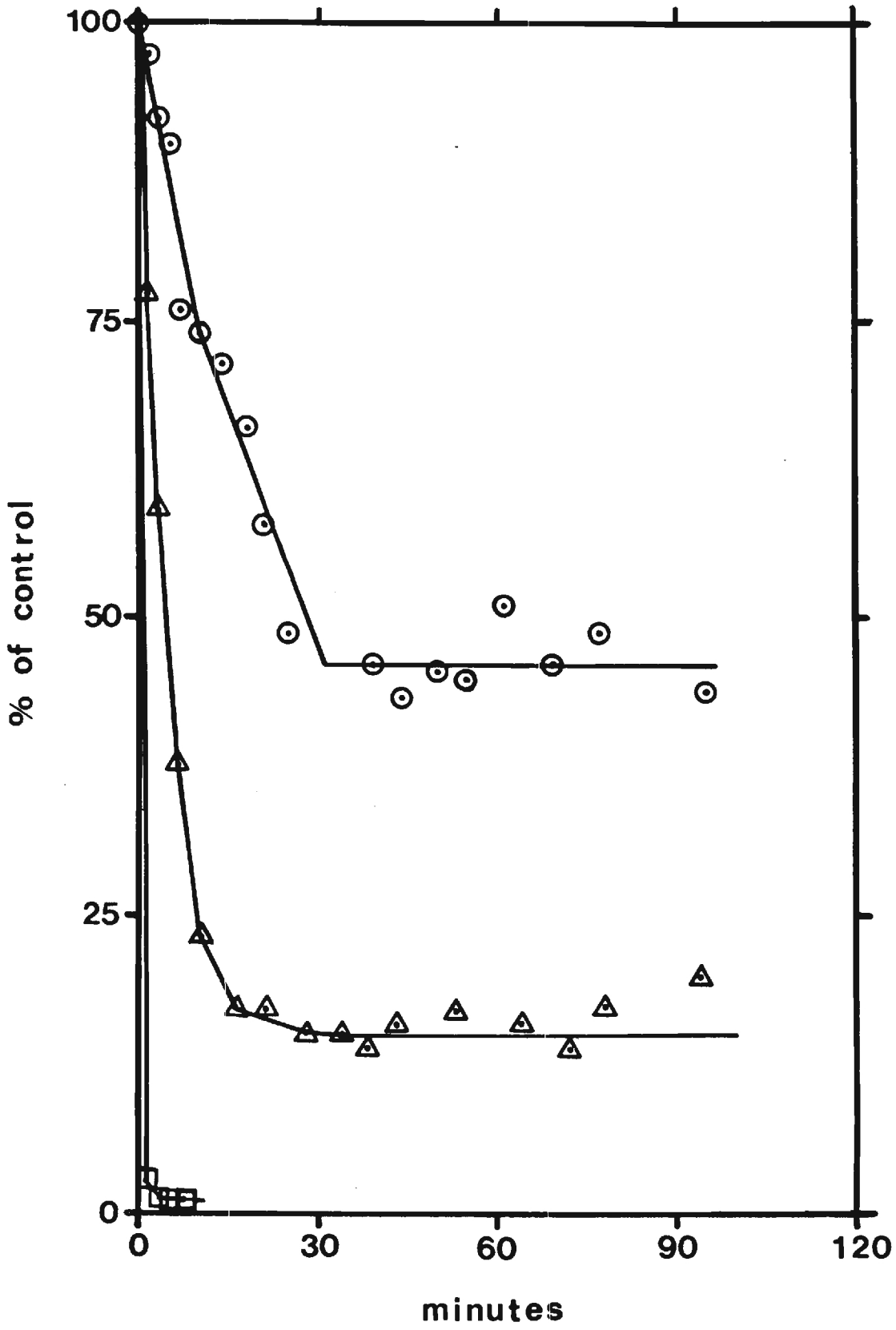

100
75
50
25
10
7.5
5.0
2.5
1.0
0.75
0.5
$10^3 \times (A_\infty - A_t)_{425}$
0
60
120
180
minutes


ln |A∞ − At|
−4
−6
−8
−10
5
10
15
seconds




2.5
1.0
.75
.5
.25
.1
0
120
240
minutes
$10^2 (A_\infty - A_t)_{420}$




100
75
50
25
0
% of control
0
30
60
90
120
minutes